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(57) Abstract

The present invention relates to the use of substances with oxytocin activity in order to improve cell regeneration. It also relates to a pharmaceutical composition comprising at least one substance with oxytocin activity in order to improve cell regeneration.

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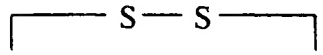
USE OF SUBSTANCES WITH OXYTOCIN ACTIVITY IN ORDER TO CREATE CELL REGENERATION

The present invention relates to the use of substances with oxytocin activity in order to create cell regeneration. It also relates to a pharmaceutical composition comprising at least one substance with oxytocin activity in order to create cell regeneration.

Background of the invention

Oxytocin was one of the first peptide hormones to be isolated and sequenced. It is a nonapeptide with two cysteine residues that form a disulfide bridge between positions 1 and 6 and corresponds to the formula



For a long time the only effects attributed to oxytocin were its stimulating effects on milk ejection and uterine contractions, but in the past decades it has been shown that oxytocin exerts a wide spectrum of effects within the central nervous system, CNS. It has been suggested that oxytocin participates in the control of memory and learning processes and of various types of behaviour such as feeding, locomotion, as well as maternal and sexual behaviour. Oxytocin is also suggested to participate in the control of cardiovascular functions, thermoregulation, pain threshold and fluid balance. There is also evidence that oxytocin is involved in the control of various immunological processes. It has recently been demonstrated that oxytocin injections cause a lowering of blood pressure and increased weight gain - long lasting effects after repetitive administration. As a central stimulating substance oxytocin plays an important role in the interaction between mother and progeny in mammals. The products may also be used prophylactic in young human beings e.g. already in new born babies or young children to prevent the development of diseases later on in life

which diseases are dependent on stress conditions during the fetal life. Such conditions may be heart/vessel diseases such as stroke, heart infarct, hypertension, and diabetes.

There are different processes described for the synthetical production of oxytocin; commercial processes are for instance described in US patents 2,938,891 and 3,076,797.

In the human body oxytocin is produced in the paraventricular nucleus, PVN, and the supraoptic nucleus, SON, of the hypothalamus. It differs by only two amino acids from vasopressin, which is also produced in these nuclei. The magnocellular oxytocinergic neurons of the SON and PVN send Parvocellular neurons that originate in the PVN project into multiple areas within CNS. The oxytocin-producing cells are innervated by cholinergic, catecholaminergic as well as peptidergic neurons. The presence of oxytocin in different tissues outside the brain, such as the uterus, ovaries, testis, thymus, adrenal medulla and pancreas has been demonstrated and oxytocin is suggested to exert local effects in these organs.

A parallel secretion of oxytocin into the brain regions and into the circulation occurs in response to some stimuli such as suckling, but other stimuli can cause separate activation of oxytocinergic neurons, terminating in the brain or the pituitary.

In this context oxytocin refers, whenever applicable, in addition to oxytocin also to precursors, metabolic derivatives, oxytocin agonists or analogues displaying the same properties.

There are several oxytocin derivatives, i.e. compounds with a structure similar to that of oxytocin. There are preliminary indications that other oxytocin derivatives than oxytocin could give the cell regeneration effects of oxytocin as well as parts of the oxytocin molecule. No publications describe the use of other oxytocin derivatives than oxytocin or parts of the oxytocin molecule to improve cell regeneration.

In experiments it has been shown that oxytocin by way of a central action increases the activity of the central α_2-receptors in rats. These receptors have an inhibitory action and counteracts the activating aspects of noradrenalin in the brain which are mainly mediated via α_1-receptors, which activate cyclic AMP. When α_2-receptor stimulation dominates over α_1-receptor stimulation, activity is exchanged by relaxation and energy is shunted towards growing and healing, i.e. is not used for stress or muscular contraction and activity. As a consequence parasympathetic nerve tone dominates over sympathetic nervous tone and the musculature is relaxed. It can be presumed that oxytocin exerts a similar effect also in humans. During breast feeding - a situation characterized by repetitive oxytocin secretion - all the effects observed in experimental animals following repeated oxytocin administration are seen. It is not known how the effect by oxytocin on α_2-receptors is mediated, but probably not by a classical oxytocin receptor mediated effect.

The effect of oxytocin can be extended or strengthened by administration in combination with drugs increasing the release of oxytocin and/or the number or affinity of receptors, such as estrogen, or drugs having an α_2-agonistic effect, such as clonidine.

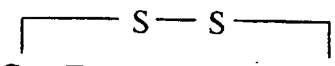
It has now turned out that oxytocin stimulates fetal growth (Example 1), enhances ovarian maturation and function (Example 2), i e an effect against sterility which is helpful in fertilisation *in vitro* and *in vivo* and to increase the sperm and egg production, stimulates recovery of peripheral and central neuropathies (Example 3), stimulates human dermal dendritic cells (Example 4), stimulates dermal keratinocytes (Example 5), stimulates the cortical bone thickness (Example 6), reduces apoptosis (Example 7), protects from phorbol ester-and oxazolone-induced inflammation (Example 8), has effects on aged skin and barrier formation (Example 9), and reduces cell energy consumption (Example 10). These Examples indicate that oxytocin or that substances with oxytocin activity may be used for cell regeneration.

Summary of the invention

The present invention relates to the use of a substance with oxytocin activity in order to improve cell regeneration. The invention also relates to a pharmaceutical composition comprising an effective concentration of at least one substance with oxytocin activity in mixture or otherwise together with at least one pharmaceutically acceptable carrier or excipient. Such a pharmaceutical composition could be used in order to improve cell regeneration.

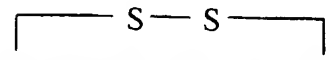
Detailed description of the invention

An object of the present invention is the use of a substance with an oxytocin like activity in order to create cell regeneration. Examples of substances with oxytocin activity are the following compounds:



Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

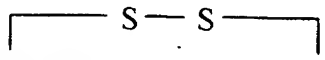
Oxytocin



Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ile-Gly-NH_2

Mesotocin

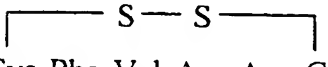
i.e. V is Tyr, W is Ile, X is Gln, Y is Ile, and Z is Gly in Claim 2 and 4



Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-NH_2

Isotocin

i.e. V is Tyr, W is Ile, X is Ser, Y is Ile, and Z is Gly in Claim 2 and 4



Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH_2

Annetocin

i.e. V is Phe, W is Val, X is Arg, Y is Thr, and Z is Gly in Claim 2 and 4

Annetocin has been isolated from the earthworm, as described in Oumi T, Ukena K, Matsushima O, Ikeda T, Fujita T, Minakata H, Nomoto K, Annetocin: an oxytocin-related peptide isolated from the earthworm, Eisenia foetida, Biochem Biophys Res Commun 1994, Jan 14; 198(1): 393-399. Other substances with oxytocin activity could also be used, such as naturally occurring or artificially modified variants, analogues, and derivatives of oxytocin, mesotocin, isotocin, and annetocin. Such substances could be obtained by addition, insertion, elimination, or substitution of at least one amino acid in these hormones. By substance with an oxytocin like activity is also understood precursors, metabolites such as metabolic derivatives e.g. metabolic degradation products, agonists, or analogues of the substances mentioned herein displaying the same properties. Metabolic derivatives or metabolic degradation products may be oxytocin like peptides e.g. with nine amino acids such as oxytocin, mesotocin, isotocin, and annetocin from which one or more amino acids has been deleted from either or both ends of the molecule. Preferably one, two or three amino acids may have been deleted from the carboxy terminal end i.e. Gly only, Gly and Leu, or Gly, Leu, and Pro. Preferably one, two or three amino acids may have been deleted from the amino terminal end i.e. Cys only, Cys and Tyr, or Cys, Tyr, and Ile. Preferably one, two or three amino acids may have been deleted both from the carboxy terminal end i.e. Gly only, Gly and Leu, or Gly, Leu, and Pro, and one, two or three amino acids may have been deleted from the amino terminal end i.e. Cys only, Cys and Tyr, or Cys, Tyr, and Ile.It could be ascertained that these variants are analogues of oxytocin, mesotocin, isotocin or annetocin by immunological methods, e.g. RIA (radio-immunoassay), IRMA (radiometric methods), RIST (radioimmunosorbent test), RAST (radioallergosorbent test).

As mentioned above there are indications that subfragments of the oxytocin molecule could give cell regeneration. Preferably, these subfragments of the oxytocin

molecule is made by deletions of amino acids outside the disulfide bridge. Examples of subfragments of the oxytocin molecule are the following compounds:

S — S

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-NH_2

SEQ ID NO: 1

i.e. V is Tyr, W is Ile, X is Gln, Y is nothing, and Z is nothing in Claim 2 and 4

S — S

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-NH_2

SEQ ID NO: 2

i.e. V is Tyr, W is Ile, X is Gln, Y is Leu, and Z is nothing in Claim 2 and 4

There is also a possibility to create new compounds with oxytocin activity by means of computer simulation. Methods for computer simulation are known by a person skilled in the art, e.g. as described in EP 0660 210 A2. Seven new compounds have been created by means of computer simulation, namely the following peptides:

S — S

Cys-Tyr-Val-Thr-Asn-Cys-Pro-Leu-Gly-NH_2

SEQ ID NO: 3

i.e. V is Tyr, W is Val, X is Thr, Y is Leu, and Z is Gly in Claim 2 and 4

S — S

Cys-Tyr-Hoph-Thr-Asn-Cys-Pro-Val-Gly-NH_2

SEQ ID NO: 4

i.e. V is Tyr, W is Hoph, X is Thr, Y is Val, and Z is Gly in Claim 2 and 4

S — S

Cys-Tyr-Phe-Cit-Asn-Cys-Pro-Leu-Gly-NH_2

SEQ ID NO: 5

i.e. V is Tyr, W is Phe, X is Cit, Y is Leu, and Z is Gly in Claim 2 and 4

S — S

Cys-Tyr-Cha-Arg-Asn-Cys-Pro-Hos-Ala-NH_2

SEQ ID NO: 6

i.e. V is Tyr, W is Cha, X is Arg, Y is Hos, and Z is Ala in Claim 2 and 4

S — S

Cys-Tyr-Val-Daba-Asn-Cys-Pro-Daba-Ala-NH_2

SEQ ID NO: 7

i.e. V is Tyr, W is Val, X is Daba, Y is Daba, and Z is Ala in Claim 2 and 4

S — S

Cys-Tyr-Hoph-Daba-Asn-Cys-Pro-Cit-Ala-NH_2

SEQ ID NO: 8

i.e. V is Tyr, W is Hoph, X is Daba, Y is Cit, and Z is Ala in Claim 2 and 4

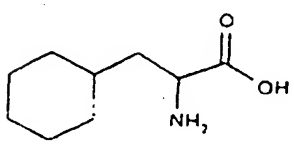
S — S

Cys-Tyr-Phe-Arg-Asn-Cys-Pro-Val-Ala-NH_2

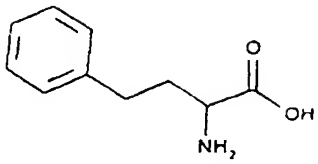
SEQ ID NO: 9

i.e. V is Tyr, W is Phe, X is Arg, Y is Val, and Z is Ala in Claim 2 and 4,

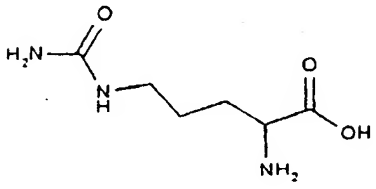
wherein Cha stands for cyclohexylalanine,



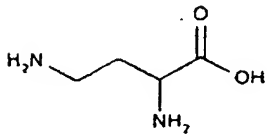
Hoph stands for homophenylalanine,



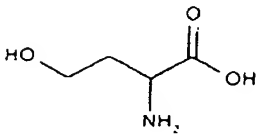
Cit stands for citruline,



Daba stands for diaminobutyric acid, and



Hos stands for homoserine.



The invention also relates to the peptides mentioned above in both D- and L-form. Especially the invention relates to the L-form. By inversion of the peptide sequence thereof, the D-form could be converted to the L-form. The effect of the D- and L-forms are the same. These and the peptides above can be produced by methods known to a person skilled in the art, e.g. according to Merrifield, P.B., "Solid Phase Synthesis", *Angew. Chemie*, **1985**, No. 97, p. 801.

It could be ascertained that compounds have oxytocin effect by tests, such as described in the publication by Cassoni, P. below and the tests according to the Examples that follow.

By the expression "cell regeneration" we understand recovery of a human or animal body by a controlled and helpful generation of existing and new cells as well as cell maturation in order to replace damaged cells. The generation of existing cells may imply vitalising of the whole cell and growth stimulating thereof for example of outgrowths such as dendrites and axones on nerve cells. Thus, dystrophic conditions of nerves and muscles may be treated e.g. multiple sclerosis (MS), and muscle rheumatism. Cassoni, P., Sapino, A, Fortunati, N., Munaron, L., Chini, B., and Bussolati, G. In contrast, oxytocin inhibits the proliferation of MDA-MB231 human breast-cancer cells *via* cyclic adenosine monophosphate and protein kinase A. *Int. J. Cancer*: **72**, 340-344 (1997) describes the inhibition of breast-cancer cells but does not describe how damaged cells in the body are replaced.

The generation of new cells and cell maturation could also be important in the healing process after a heart attack or stroke, atrophies, degenerative diseases such as Parkinson's disease and Alzheimer's disease, psoriasis, osteoporosis, after transplantations, balance disturbances, a rupture of the uterus, the liver, a kidney, in order to improve female infertility and to recover proliferation of blood cells (e.g. erythrocytes, leukocytes, and lymphocytes) e.g. after a cancer therapy, in order to increase the weight in cattle, in order to increase the human senses (e.g. to improve damaged sight, hearing, olfactory sense, and taste as a result of aging), in order to relieve incontinence problems, in cosmetic applications in order to rejuvenate skin and counteract wrinkles as a result of aging, preoperatively to increase the healing capacity, and to assist in the growth of hair and nails.

Another object of the invention is a pharmaceutical composition comprising an effective concentration of at least one substance with oxytocin activity in mixture or otherwise together with at least one pharmaceutically acceptable carrier or excipient.

Examples of substances with oxytocin activity are mesotocin, isotocin, annetocin, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

The pharmaceutical compositions according to the invention may contain substances that extend or strengthen the effects of oxytocin. Such substances could increase the release of oxytocin and/or the number or affinity of receptors, such as estrogen, or drugs having an α_2-agonistic effect, such as clonidine.

The pharmaceutical compositions are prepared in a manner known to a person skilled in the pharmaceutical art. The carrier or the excipient could be a solid, semi-solid or liquid material that could serve as a vehicle or medium for the active ingredient. Suitable carriers or excipients are known in the art. The pharmaceutical composition could be adapted to oral, parenteral, or topical use and could be administered to the patient as tablets, capsules, suppositories, solutions, suspensions or the like.

The pharmaceutical compositions could be administered orally, e.g. with an inert diluent or with an edible carrier. They could be enclosed in gelatin capsules or be compressed to tablets. For oral therapeutic administration the compounds according to the invention could be incorporated with excipients and used as tablets, lozenges, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like. These preparations should contain at least 4% by weight of the compounds according to the invention, the active ingredient, but could be varied according to the special form and could, suitably, be 4-70% by weight of the unit. The amount of the active ingredient that is contained in compositions is so high that a unit dosage form suitable for administration is obtained.

The tablets, pills, capsules, lozenges and the like could also contain at least one of the following adjuvants: binders such as microcrystalline cellulose, gum tragacanth or gelatin, excipients such as starch or lactose, disintegrating agents such as alginic acid, Primogel, corn starch, and the like, lubricants such as magnesium stearate or

Sterotex, glidants such as colloidal silica dioxide, and sweetening agents such as saccharose or saccharin could be added or flavourings such as peppermint, methyl salicylate or orange flavouring. When the unit dosage form is a capsule it could contain in addition of the type above a liquid carrier such as polyethylene glycol or a fatty oil. Other unit dosage forms could contain other different materials that modify the physical form of the unit dosage form, e.g. as coatings. Accordingly, tablets or pills could be coated with sugar, shellac or other enteric coating agents. A syrup could in addition to the active ingredient contain saccharose as a sweetening agent and some preservatives, dyes and flavouring agents. Materials that are used for preparation of these different compositions should be pharmaceutically pure and non-toxic in the amounts used.

For parenteral administration the compounds according to the invention could be incorporated in a solution or suspension. Parenteral administration refers to the administration not through the alimentary canal but rather by injection through some other rute, as subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, intravenous, intranasal, intrapulmonary, through the urinary tract, through eye drops, rectal or vaginal (e.g. as a suppository, a vagitorium, a cream or an ointment), through the lactiferous tract in cattles, into an organ such as bone marrow, etc. Bone marrow may also be treated *in vitro*. These preparations could contain at least 0.1% by weight of an active compound according to the invention but could be varied to be approximately 0.1-50% thereof by weight. The amount of the active ingredient that is contained in such compositions is so high that a suitable dosage is obtained.

The solutions or suspensions could also comprise at least one of the following adjuvants: sterile diluents such as water for injection, saline, fixed oils, polyethylene glycols, glycerol, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol or methyl paraben, antioxidants such as ascorbic acid or sodium bisulfite, chelating agents such as ethylene diamine tetraacetic acid, buffers such as acetates, citrates or phosphates, and agents for adjustment of the tonicity such as so-

dium chloride or dextrose. The parenteral preparation could be enclosed in ampoules, disposable syringes or multiple dosage vessels made of glass or plastic.

For topical administration the compounds according to the invention could be incorporated in a solution, suspension, or ointment. These preparations could contain at least 0.1% by weight of an active compound according to the invention but could be varied to be approximately 0.1-50% thereof by weight. The amount of the active ingredient that is contained in such compositions is so high that a suitable dosage is obtained. The administration could be facilitated by applying touch, pressure, massage, heat, warms, or infrared light on the skin, which leads to enhanced skin permeability. Hirvonen, J., Kalia, YN, and Guy, RH. Transdermal delivery of peptides by iontophoresis, *Nat Biotechnol* 1996 Dec; **14(13)**: 1710-1713 describes how to enhance the transport of a drug via the skin using the driving force of an applied electric field. Preferably, iontophoresis is effected at a slightly basic pH.

Other administration forms are inhalation through the lungs, buccal administration via the mouth and enteral administration via the small intestine that could be effected by means known by a person skilled in the art.

All publications mentioned herein are hereby incorporated by reference. By the expression "comprising" we understand including but not limited to. Thus, other non-mentioned substances, additives or carriers may be present.

Short description of the Figures

Figure 1. Maternal net weight (total body weight - weight of the uterus including fetal and placental tissues) gain during the first 14 and 20 days of gestation, in food restricted and ad libitum fed, NaCl- and oxytocin-treated dams. Effect of nutrition ($p<0.001$), effect of treatment ($p=0.06$), effect of day of gestation (ns), no significant interactions. The boxes indicate SEM and the bars SD.

Figure 2. Parametrial adipose tissue, on gestational days 14 and 20, in food restricted and ad libitum fed, NaCl- and oxytocin-treated dam. Effect of nutrition ($p<0.001$), effect of treatment (ns), effect of day of gestation (ns), interaction (day of gestation x treatment) ($p<0.05$), no other significant interactions. *Significant ($p<0.05$) difference between ad libitum fed oxytocin- and NaCl-treated groups on gestational day 20. The boxes indicate SEM and the bars SD.

Figure 3. Retroperitoneal adipose tissue, on gestational days 14 and 20, in food restricted and ad libitum fed, NaCl- and oxytocin-treated dams. Effect of nutrition ($p<0.001$), effect of treatment (ns), effect of day of gestation (ns), interaction (day of gestation x treatment) ($p<0.05$), no other significant interactions. *Significant ($p<0.05$) difference between ad libitum fed oxytocin- and NaCl-treated groups on gestational day 20. The boxes indicated SEM and the bars SD.

The invention will be illuminated by the following Examples, which are only intended to illuminate and not restrict the invention in any way.

Examples

Example 1 - Stimulation of fetal growth

Materials and methods

Animals

Nullipara female Sprague-Dawley rats (body weight ± 12 g) were obtained from B&K Universal AB, Sollentuna, Sweden. Animals were single housed in cages in temperature (20 ± 1°C)- and humidity (45-55%)-controlled rooms, illuminated from 0600 h to 1800 h. The diet was a pelleted ration (Lactamin, Vadstena, Sweden). The animals had free access to tap water. Body weight of dams were monitored weekly during the first four weeks and on gestational days 1, 14 and 20 and on lactational

day 1 and 20. Body weight of pups were recorded on lactational days 1, 5, 10, 15 and 20. The study was approved by the Stockholm Ethical Committee for Experiments in Animals.

Animal experiments

At arrival, animals were divided into two groups, one ad libitum fed and one food restricted. The food restricted animals obtained 70% of the average food consumed by the ad libitum fed animals throughout the experiment. After four weeks, the rats were mated and considered to be pregnant (gestational day 1); the morning sperm was found in their vaginal smear. Ad libitum fed and food restricted pregnant rats were divided into two subgroups, oxytocin treated and control. Oxytocin-treated animals were injected once a day with oxytocin (1 mg/kg body weight) while control animals were injected with saline (NaCl 0.9%) subcutaneously, during gestational day 1-5. Ad libitum fed and food restricted animals were killed on gestational days 14 and 20, and groups of ad libitum fed dams and pups were killed on lactational day 20. Pregnant rats delivered their pups late in the afternoon on gestational day 23. The day after was considered as lactational day 1. On that day, the litter was culled to 8 pups/dam. Virginal, ad libitum fed and food restricted, rats were killed at the time corresponding to day 1 of gestation for the mated rats. The following 12 groups were thus included in the experiment: NaCl-treated, virginal, ad libitum fed (n=8), NaCl-treated, virginal, food restricted (n=8), NaCl-treated, gestational day 14, ad libitum fed (n=4), NaCl-treated, gestational day 14, food restricted (n=8), NaCl-treated, gestational day 20, ad libitum fed (n=10), NaCl-treated, gestational day 20, food restricted (n=10), NaCl-treated, lactational day 20, ad libitum fed (n=4), oxytocin-treated, gestational day 14, ad libtum fed (n=4), oxytocin-treated, gestational day 14, food restricted (n=9), oxytocin-treated, gestational day 20, ad libtium fed (n=12), oxytocin-treated, gestational day 20, food restricted (n=13), oxytocin-treated, lactational day 20, ad libitum fed (n=3).

Maternal trunk blood was collected by decapitation. Maternal adipose tissue from the parametrial and retroperitoneal region, the liver, individual fetuses and placentas (only gestational day 20), were dissected out and weighed. Immediately after decapitation, trunk blood was collected in chilled plastic tubes containing 10 IU/mL heparin (Lövens Läkemedel, Malmö, Sweden) and 500 IU/mL aprotinin (Bayer AB, Stockholm, Sweden). The samples were centrifuged immediately, and the plasma was harvested and frozen (-20°C).

Analysis of blood samples

Oxytocin was determined with radioimmunoassay (RIA), after purification of plasma (500 µl) on Sep-Pak C18 cartridges (Water Corporation, Milford, MA, USA) as described by Marchini G, Lagercrantz H, Uvnäs-Moberg K. Plasma gastrin i newborn infants and their relationship tp catecholamines. J Dev Physiol 1990, 14:147-155, and dissolved in 500 µl assay buffer (0.05 M phosphate buffert with 0.1% BSA, pH 7.5). The concentration of oxytocin was determined in the purified plasma (100 µl) incubated with 50 µl of the antibody A19 (Euro-Diagnostica AB, Malmö, Sweden) at +4°C for 24h. 50 µl of tracer (^{125}I)-Tyr^2-oxytocin, specific activity 2200 Ci/mmol (DuPont NEN Research Products, Boston, MA, USA) was added and the incubation continued at +4°C for 48 h. Antibody and (^{125}I)-Tyr^2-oxytocin were diluted in 0.05 M phosphate buffer with 0.1% BSA. Cross-reactivity with arginine-vasotocin was 0.01%, with lysine-vasopressin <0.01% and with arginine-vasotocin 0.1%. The bound fraction was separated from the unbound by incubation of the samples with a second rabbit antibody Decanting Suspension 3 (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). The samples were centrifuged (1700 x g) for 10 min, the supernatant decanted and the radioactivity in the precipitate determined. The limit of detection was 3.2 pmol/l. The intra- and inter-assay coefficients of variation were 18% and 26%, respectively.

Statistical analysis

Results are presented as mean ± SD. The effect of pregnancy in ad libitum fed and food restricted control animals was assessed by two-way ANOVA, having nutrition (ad libitum fed vs food restricted) and day of gestation (virginal, day 14 and day 20) as components of variation. The effect of oxytocin-treatment was assessed by a three-way ANOVA, having nutrition (ad libitum fed and food restricted), treatment (oxytocin vs saline) and day of gestation (day 14 vs day 20) as components of variation. If a significant interaction was found, planned comparisons were used to assess differences between oxytocin and corresponding NaCl treated group. Differences in proteolytic activity and differences in postnatal pup weight, between oxytocin and NaCl treated groups, were assessed by Student's t-test for independent means. Results were considered significant when $p<0.05$.

Results

Oxytocin-treated rats tended ($p=0.06$) to have a smaller net weight gain during gestation than their NaCl-treated counterparts (Figure 1). On gestational day 20, ad libitum fed oxytocin-treated rats contained relatively (% of net body weight) less parametrial (Figure 2) and retroperitoneal (Figure 3) adipose tissue compared with saline treated rats, while earlier in pregnancy, there was no difference in the amount of parametrial and retroperitoneal adipose tissue between oxytocin and saline treated rats.

Fetuses and placentas from ad libitum fed oxytocin treated dams were larger on gestational day 20 and the birthweight was higher, compared with conceptus and offspring from NaCl-treated rats (Table 1). No further changes in postnatal body weights between pups from ad libitum fed oxytocin and NaCl-treated dams was observed. In contrast to the ad libitum fed group, there was no effect of oxytocin on fetal weight in the food restricted group (Table 1).

Table 1. Reproductive outcome of ad libitum fed and food restricted animals. Values are mean ± SD.

	Ad libitum fed		Food restricted	
	NaCl	Oxytocin	NaCl	Oxytocin
Fetal/pup weight (g)				
Gestational day 14[a]	0.067 ± 0.011	0.069 ± 0.009	0.058 ± 0.010	0.060 ± 0.014
Gestational day 20[a]	2.137 ± 0.168	2.275 ± 0.213[b]	1.914 ± 0.274	1.879 ± 0.255
Lactational day 1	7.291 ± 0.232	7.619 ± 0.473[b]		
Lactational day 5	14.425 ± 0,512	14.783 ± 0.958		
Lactational day 10	26.391 ± 1.285	26.648 ± 1.565		
Lactational day 15	39.350 ± 2.315	40.417± 2.489		
Lactational day 20	52.321 ± 3.106	54.021 ± 3.253		
Placental weight (g)				
Gestational day 20[a]	0.476 ± 0.086	0.530 ± 0.089[b]	0.429 ± 0.058	0.412 ± 0.067

[a]Significant difference between ad libitum fed and food restricted groups

[b]Significant difference between NaCl and oxytocin treated groups

Discussion

Oxytocin had small but significant beneficial effects on fetal growth in ad libitum fed animals, but no further effect on postnatal growth during the lactational period. It is conceivable that other doses of oxytocin, way or ways of administration could further enhance fetal growth. The effects of oxytocin could probably vary depending

on when during gestation it is administered. According to the present invention, oxytocin was injected during the first five days of gestation, a period when the fertilised ovum is not yet implanted. Therefore, the stimulatory effects of oxytocin on fetal growth is likely to be explained by altered maternal adaptions to pregnancy, having positive effects on, for example, placental nutrient transfer capacity or the ability of the mother to mobilise her body stores in late pregnancy.

Example 2 - Enhancement of ovarian maturation and function

Neurotrophins (NT) play a central role in the differentiation and survival of neural population within both the central and periferal nervous system. At least four different NTs are found in the ovary.

Materials and methods

Animals

This animal model is as described in Petersson M, Alster P, Lundeberg T. and Uvnäs-Moberg K. Oxytocin Causes a Long-Term Decrease of Blood Pressure in Female and Male Rats. Physiology & Behavior, Vol. 60, No. 5, pp. 1311-1315, 1996, but only female rats were used. Experiments were performed in 2 series. Female Sprague-Dawley rats (230-250 g) were used for sc injection The animals arrived at least 3 weeks before experiments and were housed 5 per cage, with free access to food and water. The light schedule was a 12/12h light/dark cycle. The ambient temperature was 20 ± 2°C. The stage of the estrous cycle was determined by microscopic examination of vaginal smears.

Experimental design

NaCl, oxytocin 0.01, 0.1, and 1 mg/kg (Ferring, Malmö, Sweden) and NaCl again were given sc for consecutive 5-day periods to female rats. Five-day treatment peri-

ods were chosen to cover the duration of the estrous cycle in the rats. Drugs were dissolved in physiological saline and injected in a volume of 1 mL/kg. Control animals received NaCl sc (1 mL/kg) during the entire treatment period.

Results

The results show that in comparison with controls, the oxytocin treated rats had an increased expression (through NGF gene stimulation) and synthesis as demonstrated by PCR, Northern blot, and autoradiography of the NGF trkA receptor (22% ≥ controls) and NGF (26% ≥ controls) in the ovary. This results in increased folliculogenesis and subsequent follicular development (Ojeda and Dissen, *Ovarian development*, p. 32 (1994)).

Example 3 - Recovery of peripheral and central neuropathies

Materials and methods

Animals

This animal model is as described in Petersson M, Alster P, Lundeberg T. and Uvnäs-Moberg K. Oxytocin Causes a Long-Term Decrease of Blood Pressure in Female and Male Rats. Physiology & Behavior, Vol. 60, No. 5, pp. 1311-1315, 1996. Experiments were performed in 2 series. Female and male Sprague-Dawley rats (230-250 g) were used for sc injection and male Sprague-Dawley rats (335-375 g) for intracerebroventricular (icv) injection (B&K Universal AB, Sollentuna, Sweden). The animals arrived at least 3 weeks before experiments and were housed 5 per cage, except animals provided with icv cannulas that were housed individually, with free access to food and water. The light schedule was a 12/12h light/dark cycle. The ambient temperature was 20 ± 2°C. In female rats, the stage of the estrous cycle was determined by microscopic examination of vaginal smears.

Surgery for icv injections

Following anesthesia with pentobarbitalnatrium (Apoteksbolaget, Sweden), 50 mg/kg injected intraperitoneally (ip), the skull was uncovered and a guide cannula (21 g) was stereotactically fixed to the skull by acrylic dental cement. The coordinates were 1.00 mm posterior and 1.30 mm lateral to the bregma. The guides reached, but did not penetrate, the dura mater, with the needle tip in the lateral ventricle. The animals were allowed 1 week of recovery after the operation. At the end of the experiment, the placement of the guide cannula was checked by injection of 2 µl of Toluidine Blue.

Experimental design

NaCl, oxytocin 0.01, 0.1, and 1 mg/kg (Ferring, Malmö, Sweden) and NaCl again were given sc for consecutive 5-day periods to male and female rats. Five-day treatment periods were chosen to cover the duration of the estrous cycle in female rats. Drugs were dissolved in physiological saline and injected in a volume of 1 mL/kg. Control animals received NaCl sc (1 mL/kg) during the entire treatment period.

In a second series of experiments, oxytocin was given icv (1 µg/kg) in a volume of 5 µl during 5 days to male rats provided with chronic icv cannulas. Control animals received 5 µl saline icv.

The role of NGF

Nerve growth factor (NGF), a powerful agent for the growth, differentiation and regeneration of lesioned cells of the central and peripheral nervous system, has in recent years been indicated as a potential therapeutic agent capable of reversing the process of cell damage in neurodegenerative events in man. Since NGF does not cress the blood brain barrier and central NGF administration requires invasive surgical procedures, the discovery of substances modulating in vivo NGF synthesis in the

brain will be extremely useful for a possible clinical use of NGF. Also, NGF is a target-derived growth factor that plays a crucial role in growth and differentiation of peripheral sensory and sympathetic neurons. NGF has been shown to promote survival not only of surgical and chemical injured peripheral nerve cell damage but also of diabetes neuropathies, leprosy, post-ischemic coronary innervation, and erectile function. As a result of these observations NGF has likely a role in the treatment of peripheral neuropathies. Because numerous cells are able to produce local and circulating NGF a possible strategy is to identify compounds that induce up-regulation of NGF in vivo.

Central effects

The aim of the present study was to analyse if the content of NGF in the brain of adult rats can be affected by peripheral (subcutaneous, sc) administration of oxytocin. The NGF concentrations in the hypothalamus and hippocampus were analysed by the use of a sensitive immunoenzymatic assay for NGF.

Table 2 shows the results that were obtained regarding brain NGF levels (pg/g tissue) in the hippocampus and hypothalamus of rats injected sc with oxytocin compared to the saline control animals.

Table 2. Brain NGF levels (pg/g tissue) of rats injected sc with oxytocin.

Part of the brain	Treated with saline	Treated with oxytocin
Hippocampus	4312 ± 213	5674 ± 389
Hypothalamus	447 ± 66	1543 ± 455

The results in Table 2 suggest that sc injection of oxytocin can modulate the NGF levels in the brain.

Peripheral effects

To elucidate the peripheral effects of sc oxytocin, two models of peripheral neuropathies in rats were used: one involving the peripheral sensory nervous system (by the use of capsaicin) and one involving the peripheral sympathetic nervous system (by the use of 6-hydroxydopamine, 6-OHDA). In the present study we have used these animal models to investigate whether exogenous administration of oxytocin influences the changes induced by 6-OHDA on the iris/hind paw and of capsaicin on the hind paw. The Tables below show the results that were obtained regarding peripheral NGF levels (pg/g tissue) in iris (Table 3) and hind paw (Table 4 for 6-OHDA and Table 5 for capsaicin) of rats injected sc with oxytocin compared to the saline control animals.

Table 3. Peripheral NGF levels (pg/g tissue) of rats injected sc with oxytocin in the iris compared to saline control animals, by the use of 6-OHDA.

	NGF pg/g
6-OHDA + saline	834 ± 115
6-OHDA + oxytocin	9844 ± 1206

Table 4. Peripheral NGF levels (pg/g tissue) of rats injected sc with oxytocin in the hind paw to saline control animals, by the use of 6-OHDA.

	NGF pg/g
6-OHDA + saline	756 ± 223
6-OHDA + oxytocin	6711 ± 987

Table 5. Peripheral NGF levels (pg/g tissue) of rats injected sc with oxytocin in the hind paw to saline control animals, by the use of capsaicin.

	NGF pg/g
Capsaicin + saline	1039 ± 438
Capsaincin + oxytocin	1432 ± 519

To elucidate if the treatment had any physiological significance, the hot plate response was used. Table 6 shows to the latency to lick (startle response) for animals treated with capsacin + saline and capsaicin + oxytocin, respectively.

Table 6. Latency to lick (startle response) (in seconds) for rats treated with oxytocin in the hind paw to saline control animals, by the use of capsaicin.

	Latency to lick (sec)
Capsaicin + saline	43 ± 15
Capsaicin + oxytocin	32 ± 9

The results in Table 3-6 show that a repeated administration of oxytocin increases the NGF content in the lesioned tissue and this may have functional implications.

Example 4 - Stimulation of human dermal dendritic cells

Materials and methods

The tetanus toxoid (TT) model was used that was described by Demotz et al. J Immunol 143:3881-3886. In the present study we set out to investigate the effects of oxytocin on a tetanus toxoid driven T cell proliferation model (proliferation assay). We used TT as a model antigen because most Swedish patients have a history of vaccination for TT and possess circulating TT specific memory T cells. To test the effects of oxytocin, one group of dendritic cells were preincubated with oxytocin and one with saline. Resting CD4 positive T cells were used as responders. The dendritic cells were pulsed with TT for 2h at 37°C and incubated with 5x100,000

responder cells resulting in a stimulator: responder ratio of 1:1,000, 1:100 and 1:10. Proliferative responses in these cultures were measured after 3d.

Dendritic cells

Dendritic cells belong to a family of antigen-presenting cells that are located in the T cell dependent area of lymphoid tissues. The dendritic cells are also found at the barrier zones where antigen entry into the human body may take place such as the skin, lung and gut. Recent evidence indicates a role for dermal dendritic cells in contact hypersensitivity reactions (Tse and Cooper, 1990, J Invest Derm 94:267), psoriasis (Nestle et al, 1994, J Clin Invest 94:202-209), lymphoproliferative disorders (Nestle et al, 1995, Dermatol 190:265-280). Dendritic cells have been studied for their potency for presentation of bacterial derived superantigens and soluble protein antigens to T cells (Nestle and Nickoloff, 1995, Adv Exp Med Biol 378:111-116).

Results

Table 7 shows the 3H-Thymidine Incorporation in counts per minute (cpm) as a function of the numbers of stimulator cells for dendritic cells preincubated with oxytocin compared to dendritic cells preincubated with saline.

Table 7. 3H-Thymidine Incorporation in cpm as a function of the numbers of stimulator cells for dendritic cells preincubated with oxytocin compared to dendritic cells preincubated with saline.

Numbers of stimulator cells	3H-Thymidine Incorporation (cpm)	
	Saline	Oxytocin
50	3,200	4,300
500	4,800	7,400
5,000	12,000	15,100

The results in Table 7 show that oxytocin increases the induction of dendritic cells TT specific T cell proliferation.

Example 5 - Stimulation of dermal keratinocytes

Materials and methods

The procedure of Rheinwald JG and Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331-334 (1975), was used for cultivation of human keratinocytes. This procedure used serum-free keratinocyte medium supplemented with bovine pituitary extract and epidermal growth factor; primarily cultured keratinocytes at passages 3-5 were used. Keratinocytes were then challenged with oxytocin or saline. The cell media was then analysed for TGF-b1 using enzyme-linked immunosorbent assay (sandwich ELISA).

Keratinocyte therapy

It is well known that TGF-b1 is one of the most effective growth factors stimulating the synthesis and deposition of various matrix proteins important in differentiation, morphogenesis and wound healing (Goldstein RH, Foliks CF, Pilch PF, Smith BD, and Fine A. Stimulation of collagen formation by insulin and insuline-like growth factor I in cultures of human lung fibroblasts. *Endocrinol* 124:964-970 (1989); Roberts AB and Sporn MB. In: Sporn MD and Roberts AB (eds). *The Handbook of Experimental Pharmacology, Peptide Growth Factors and their Receptors*. Springer-Verlag, Heidelberg, 1989, p. 419-472; Hill DJ, Logan A, McGarry M, De Sousa D: Control of protein and matrix-molecule synthesis in isolated bovine fetal growth-plate chondrocytes by the interactions of basic fibroblast growth factor, insulin-like growth factor-I and II, insulin and transforming growth factor-β1, *J Endocrinol* 133:363-373 (1992)). The process of cultivating keratinocytes in vivo to confluency for grafting onto patients with severe skin injuries. often thermal ones, has been as

well established (Green H, Kehinde O, and Thomas J: Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. *Proc Nat Acad Sci (USA)* 76:5665-5668, (1979)). Keratinocyte therapy, therefore, offers enormous potential for novel therapeutic approaches not only following thermal injury but also for other congenital and acquired disorders of the skin.

Results

Table 8 shows the TGF-b1 concentration in pg/mL in the cell media.

Table 8. TGF-b1 concentration (pg/mL) in cell media for keratinocytes challenged with oxytocin compared to keratinocytes challenged with saline.

	TGF-b1 concentration (pg/mL)
Keratinocytes + saline	12 ± 4
Keratinocytes + oxytocin	34 ± 8

The results in Table 8 show that oxytocin increases the production of TGF-b1 from keratinocytes.

Example 6 - Stimulation of cortical bone thickness

Materials and methods

The procedure of Östenson et al (J Diabetes Complications 1997 Nov; 11(6):319-322) was used. They studied the occurrence of osteopenia, as reflected by decreased cortical bone thickness, in a nonobese animal model of hereditary non-insulin-dependent diabetes (NIDDM) with long duration, i.e., 8-month-old Goto-Kakizaki (GK) rats. In addition, motor nerve-conduction velocity was measured in the GK rats. Age- and weight-matched Wistar rats served as controls. The GK rats displayed marked glucose intolerance, as compared to control rats, in an intraperitoneal gluco-

se tolerance test. Decreased cortical bone thickness by approximately 15%, was evident in X-ray analysis of metatarsal bones ($p << 0.001$) and humerus ($p << 0.05$) of the GK rats. Motor nerve-conduction velocity, measured in the sciatic nerve, was also decreased (by 10%) in the GK as compared with the age-matched control rats ($p << 0.05$) as a sign of peripheral neuropathy. Thus, the GK rat appears to be a model of NIDDM suitable for studies of diabetic bone disease in the absence of obesity.

In order to study the effect of oxytocin in spontaneously diabetic rats the effect of sc oxytocin and saline was investigated: 3 rats (controls) received saline and 3 rats were treated with oxytocin once a day (1 mg/kg body weight) for 5 days monthly for six months before being sacrificed. Thereafter the right humeral bones were dissected and subjected to radiogrammetry (Selby. Diabetic Med 5:423-428, 1988; Bouillon Calcif Tissue int 49:155-160, 1991). Plain X-rays were taken of the humeral bones from the rats and analysed by measuring the width and the inner noncortical width of the diaphysis of each bone by a precision caliper. A cortical thickness index was calculated by dividing the outer by the inner (cancellous) diaphyseal width.

Results

In the spontaneous diabetic rats recieving saline (control rats), the cortical bone thickness index was 1.9 ± 0.3 and in the oxytocin treated rats it was 2.1 ± 0.2. These results show that oxytocin stimulates the cortical bone thickness.

Example 7 - Reduction of sulfur mustard induced cell death (apoptosis) in keratinocytes

Materials and methods

Normal human epidermal keratinocytes were obtained as primary cultures and maintained in serum-free keratinocyte growth medium.

The effects of sulfur mustard

Sulfur mustard induces blisters in the skin via calcium-calmodulin and caspase-dependent pathways. Because sulfur mustard is a strong alkylating agent, its ability to induce DNA damage via apurinic sites and endonucleolytic activation has been advanced as one possible pathway leading to vesication.

Similar to other agents that induce DNA strand breakage, sulfur mustard stimulate the nuclear protein poly ADP-ribose polymerase, which significantly reduces the concentrations of cellular nicotin-amide adenine dinucleotide and adenosine triphosphate, a mechanism proposed to induce cell death (apoptosis).

As it has been shown that sulfur mustard may induce apoptosis in keratinocytes, keratinocytes were administered oxytocin in parallel with challenge with sulfur mustard.

Experimental design

The normal human epidermal keratinocytes were grown in tissue culture flasks to 70-80% confluency, then exposed to sulfur mustard diluted in keratinocyte growth medium, with or without oxytocin 1 mM, to final concentrations of 100 μM or 300 μM. Media was not changed for the duration of experiments. Cell viability was measured by the ability of cells to exclude trypan blue.

Results

Apoptosis is characterized by the appearance of nucleosome-sized ladders. DNA isolated from normal human epidermal keratinocytes treated with 100 μM sulfur mustard was intact which was also the case with normal human epidermal keratinocytes treated with 300 μM sulfur mustard and 1 mM oxytocin. On the other hand normal human epidermal keratinocytes treated with 300 μM sulfur mustard showed

nucleosome-sized ladders on visual inspection following analysis with agarose gel electrophoresis and ethidium bromide staining. Trypan blue exclusion at 24 h was 97% in control cells, 88% following treatment with 300 mU sulfur mustard and oxytocin and 57% in cells treated with 300 mU sulfur mustard only.

Taken together oxytocin reduces the toxicity of sulfur mustard indicating an anti-apoptotic effect.

Example 8 - Protection from phorbol ester-and oxazolone-induced inflammation.

Materials and methods

Animals

Female BALB/C mice and Crl:NMRI BR mice from Charles River were used. They were used after an adaption period of 14 d for harvesting of peritoneal macrophages, BALB/C, or for induction of allergic contact dermatitis and irritant contact dermatitis, Crl:NMRI BR.

Experimental design

For in vitro experiments, oxytocin was dissolved in RPMI 1640 (Gibco Life Technoogies, UK) supplemented with 5 or 10% heat-inactivated fetal calf serum. Penicillin, streptomycin, L-glutamine, 2-mercaptoethanol, lipopolysaccharide (LPS) from Escheria coli, oxazolone and dexametasone from Sigma and recombinant mouse interferone gamma, interleukin(IL)-1-beta, tumor necrose factor (TNF)-alfa, and IL-10 enzyme linked immunosorbent assay kits were obtained from Genzyme. RPMI 1640 was supplemented with penicillin, streptomycin, glutamine and 2-mercaptoethanol. Thioglycolate-elicited macrophages were obtained as reported by Lam et al, Can J Infect Dis 3: 94-100, 1992. Peritoneal lavage was performed using 10 mL of cold RPMI containing 5% fetal calf serum. The macrophages were washed with the same

medium and 2x 10,000,000 cells per 2 mL were cultured in 24 well plates (Nunc) with or without LPS, 10 ng per mL for cytokine proteins and nitrites, and oxytocin 100 µg per mL at 37°C in 5% CO_2, in an air humified atmosphere for 6 and 24 h. Cytokines were analysed using the commercial kits and nitrite was assayed using the standard Griess assay (Green et al, Anal Biochem 126:131-138, 1982). Tetradecanoylphorbol-13-acetate (Sigma)-induced irritant contact dermatitis was initiated by the topical application of 10 µl 0.01% TPA to the inner surface of the left ear of groups of eight mice. For topical treatment, the test area was treated with 10 µl of the compounds or vehicle DAE 244 (dimethylacetamide: acetone: ethanol; 2:4:4, vol/vol/vol) 30 min before irritation. The right ears remained without irritation and treatment. Identical treatment was performed with dexametasone as a positive control. The animals were sacrificed and the ears cut off under standard conditions 6 h after the application of the irritant. The inflammation was assessed as the increase in the ear weight (Staite et al, Blood 88:2973-2979, 1996). The efficacy of oxytocin was calculated as percentage inhibition of inflammation relative vehicle-control.

Pathophysiologic mechanisms

High concentrations of pro-inflammatory cytokines and nitric oxide are proposed to orchestrate pathophysiologic mechanisms associated with various allergic and inflammatory disorders. It is well established that after an antigen challenge or an inflammatory insult, mast cell-derived mediators such as histamine and serotonin induce vasodilation and increase permeability of blood vessels resulting in edema formation and swelling. In dinitrofluorobenzene induced hypersentivity response, expression of mRNA for interleukins are detected in the epidermis. In these conditions, keratinocytes, Langerhans cells, macrophages and lymphocytes are thought to contribute, in part, to the induced pathology through their local production of increased amounts of cytokines in the epidermis. In atopic dermatitis expression of the cytokine interferon-gamma, is proposed to perpetuate the chronic eczematous skin lesions (Therpen et al, J Allergy Clin Immunol 97:828-837,1996). There is also evidence that NO is a potential mediator of the immunologic and inflammatory reactions seen

in several dermatoses (Morita et al, J Invest Dermatol 107:549-552, 1996). Safe and efective therapies are needed for inflammatory skin disease. Several established therapies are needed for inflammatory skin disease. Several established effective topical and systemic treatments for dermatitis are available, however, all of the suffer from more or less severe side effects and some have additional inconvenient aspects that restict their use (Brethnach, Textbook of Dermatology, Blackwell,2961-3036,1992; Hay et al, Textbook of Dermatology, Blackwell, Oxford, 1391-1458, 1992; Robertson and Maibach, Topical Corticosteroids, Karger, Basel, 163-169, 1992).

The aim was to evaluate the potential anti-inflammatory effect of oxytocin using in vitro models of production of proinflammatory mediators by activated macrophages and animal models of irritant and allergic contact dermatitis. The reason is that if inflammatory processes are inhibited, cell regeneration will be promoted.

Results

Suppression of the release of cytokine in culture supernatants by LPS-activated macrophages

Macrophages incubated with oxytocin or in medium alone for 24 h did not release detectable amounts of cytokines into culture supernatants. Activation of the supernatants by LPS resulted in a significant release of IL-1-beta (32 ± 11 pg/mL, TNF-alfa (2122 ± 186 pg/mL), IL-10 (98 ± 22 pg/mL) and IFN-gamma (2118 ± 387 pg/mL) in culture supernatants. Coculture of the cells with both LPS and oxytocin inhibited the release of IL-1-beta (by 62%), TNF-alfa (by 28%), IL-10 (by 68%) and IFN-gamma (by 12%).

Inhibition of nitrite (NO metabolite) in culture supernatants of LPS-activated macrophages

Macrophages incubated with oxytocin or in medium alone for 24 h did not release detectable amounts of nitrite into culture supernatants. Activation (incubation) of the supernatants with LPS induced high levels of nitrite (8.2 ± 3.6 nitrite μM). Coculture of the macrophages with both LPS and oxytocin resulted in a significant suppression of of nitrite generation (4.3 ± 1.2 nitrite μM).

Suppression of ear inflammation caused by phorbol ester oxazolone

To evaluate the potential in vivo effect of oxytocin ear edema was induced by painting the ears of normal mice with a solution of phorbol ester. Oxytocin (1 mg per ear) and the vehicle were applied to the ears 30 min prior to the induction of contact irritation. The pinnal weight in mg in the vehicle treated group was 34.3 ± 6.1 and 17.9 ± 9.7 in the oxytocin treated group, a significant reduction. The anti inflammatory effect of oxytocin was also examined in oxazolone-induced ear inflammation. In this model, mice were sensitized with oxazolone on the shaved abdomen. Seven days later, contact hypersensitivity was elicited by applying the hapten onto the ears. The anti-inflammatory effect of topically applied oxytocin was examined 30 min after hapten application. The efficasy of treatment was assed 24 h later. Pinnal weight in mg following treatment with vehicle was 32.6 ± 7.5mg and 19.6 ± 8.7 mg following topical application with oxytocin (1 mg/ear).

Taken together our findings suggest that oxytocin exerts a protective effect against phorbol ester- and oxazolone induced ear inflammation through the suppression of proinflammatory mediator release, as suggested from the in vitro studies using activated macrophages.

Example 9 - Effects of oxytoxin treatment on aged skin and barrier formation

Materials and methods

The protocol of Hanley et al. J Invest Dermatol, 106:404-411 (1996); Hanley et al. J Clin Invest 97:2576-2584 (1996), was used.

Previous studies

Because a protective barrier is essential for life, the development of the epidermis and stratum corneum must be completed prior to birth (Komuves et al, J Invest Dermatol 1998 Sep;111(3):429-433). The epidermal permeability barrier is comprised of corneocytes embedded in a lipid enriched matrix. Recent studies, using an explant model of fetal rat skin development that closely parallels in utero development, have shown that hormones and other activators of members of the nuclear receptor family regulate permeability barrier ontogenesis by stimulating lipid metabolism and the formation of the extracellular lipid lamellae.The results of Komuves et al, demonstrate that several hormones and activators of nuclear hormone receptors regulate epidermal differentiation during fetal development, affecting key constituents of both keratohyalin granules and the cornified envelope. Thus, a variety of ligands/activators of nuclear receptors accelerate not only permeability barrier ontogenesis, but also the expression of structural proteins essential for stratum corneum formation.

Previous studies have shown that ontogeny of the epidermal permeability barrier and lung occur in parallel in the fetal rat. Gender also influences lung maturation, i.e., males exhibit delayed development. Sex steroid hormones exert opposite effects on lung maturation, with estrogens accelerating and androgens inhibiting. In a recent study, Hanley et al (1996) demonstrate that cutaneous barrier formation, measured as transepidermal water loss, is delayed in male fetal rats. Administration of estrogen to pregnant mothers accelerates fetal barrier development both morphologically and

functionally. Competent barriers also form sooner in skin explants incubated in estrogen-supplemented media in vitro. In contrast, administration of dihydrotestosterone delays barrier formation both *in vivo* and *in vitro*. Finally, treatment of pregnant rats with the androgen antagonist flutamide eliminates the gender difference in barrier formation.

Aged epidermis displays altered drug permeability, increased susceptibility to irritant contact dermatitis, and often severe xerosis, suggesting compromise of the aged epidermal barrier. To delineate the functional, structural, and lipid biochemical basis of epidermal aging, Ghadially et al, J Clin Invest 1995 May; 95(5):2281-2290 compared barrier function in young (20-30 yr) vs aged (> 80 yr) human subjects, and in a murine model. Baseline transepidermal water loss in both aged humans and senescent mice was subnormal. However, the aged barrier was perturbed more readily with either acetone or tape stripping (18 ± 2 strippings vs 31 ± 5 strippings in aged vs young human subjects, respectively). Moreover, after either acetone treatment or tape stripping, the barrier recovered more slowly in aged than in young human subjects (50 and 80% recovery at 24 and 72 h, respectively, in young subjects vs 15% recovery at 24 h in aged subjects), followed by a further delay over the next 6 d. Similar differences in barrier recovery were seen in senescent vs young mice. Although the total lipid content was decreased in the stratum corneum of aged mice (approximately 30%), the distribution of ceramides (including ceramide 1), cholesterol, and free fatty acids was unchanged. Moreover, a normal complement of esterified, very long-chain fatty acids was present. Finally, stratum corneum lamellar bilayers displayed normal substructure and dimensions, but were focally decreased in number, with decreased secretion of lamellar body contents. Thus, assessment of barrier function in aged epidermis under basal conditions is misleading, since both barrier integrity and barrier repair are markedly abnormal. These functional changes can be attributed to a global deficiency in all key stratum corneum lipids, resulting in decreased lamellar bilayers in the stratum corneum interstices. This constellation of findings may explain the increased susceptibility of intrinsically aged skin to exogenous and environmental insults.

Results

In investigating the effects of oxytocin (exchanged with estrogen in the research protocol) administration, our results indicate that oxytocin accelerates cutaneous barrier formation through an action directly on the skin. In another series of studies we set out to investigate if topical application of oxytocin could affect the total lipid content in aged skin. Daily repeated application of oxytocin for 21 days increased the total lipid content with 1.1-1.6 % in aged skin suggesting that topical application of oxytocin may have a protective effect towards exogenous and environmental insults.

Example 10 - Reduction of cell energy consumption

The aim of the present study was to investigate the effects of oxytocin on intracellular Ca^{2+}. We used the human colonic tumor cell line, HT-29 and the patch-clamp technique. Recently, Sand et al (Am J Physiol 1997 Oct; 273(4 Pt 1):C1186-C1193) used the patch-clamp technique to study the effects of carbachol (CCh) on HT-29 cells. During CCh exposure, the cells (n = 23) depolarized close to the equilibrium potential for Cl- (E(Cl-); -48 mV) and the membrane potential then started to oscillate (16/23 cells). In voltage-clamp experiments, similar oscillations in whole cell currents could be demonstrated. The whole cell conductance increased from 225 ± 25 pS in control solution to 6.728 ± 1.165 pS (means ± SE, n = 17). In substitution experiments (22 mM Cl- in bath solution, E(Cl-) = 0 mV), the reversal potential changed from -41.6 ± 2.2 mV (means ± SE, n = 9) to -3.2 ± 2.0 mV (means ± SE, n = 7). When the cells were loaded with the calcium-sensitive fluorescent dye, fluo 3, and simultaneously patch clamped, CCh caused a synchronous oscillating pattern of fluorescence and membrane potential. In cell-attached patches, the CCh-activated currents reversed at a relative membrane potential of 1.9 ± 3.7 mV (means ± SE, n = 11) with control solution in the pipette and at 46.2 ± 5.3 mV (means ± SE, n = 10) with a 15 mM Cl- solution in the pipette. High K^+ (144 mM) did not change the re-

versal potential significantly (P << or = 0.05, n= 8). In inside-out patches, calcium-dependent Cl- channels could be demonstrated with a conductance of 19 pS (n = 7). They concluded that CCh causes oscillations in membrane potential that involve calcium-dependent Cl-channels and a K^+ permeability.

Results

Using the same set up we investigated the effects of 1 mM of oxytocin. During oxytocin exposure, the cells (n = 2) depolarized close to the equilibrium potential for Cl- (E(Cl-); -48 mV) and the membrane potential then started to oscillate. In voltage-clamp experiments, similar oscillations in whole cell currents could be demonstrated. The whole cell conductance increased from 234 ± 26 pS in control solution to 6.262 ± 1.434 pS (means +/- SE, n = 2). When the cells were loaded with the calcium-sensitive fluorescent dye, fluo 3, and simultaneously patch clamped, oxytocin caused a synchronous oscillating pattern of fluorescence and membrane potential. In cell-attached patches, the oxytocin-activated currents reversed at a relative membrane potential of 2.62 In inside-out patches, calcium-dependent Cl- channels could be demonstrated with a conductance of 14 pS (n = 2). In conclusion, oxytocin reduces energy consumption.

It was found that stimulation with oxytocin increased intracellular Ca^{2+} oscillation. Oscillation in intracellular Ca^{2+} is a common pattern in different cell types. Within Ca^{2+} oscillations, the cell has the ability to modulate the response to external stimuli both with frequency and amplitude. In a study by Words et al. (Nature 319:600-602, 1986), calcium oscillations were observed in rat hepatocytes. They suggested that the frequency of the calcium transients would determine the cellular response to the hormone that initiated the calcium signal. From an energetic point of view, calcium transients decrease the intracellular calcium load and thus decrease the need for ATP-consuming calcium pumps in the membrane of calcium-storing organelles to be activated. It is also well known that sustained increase of $[Ca^{2-}]$ can be damaging to the cell (Shuttleworthe, T.J. J. Exp. Biol. 200:303-314, 1997).

SEQUENCE LISTING

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Claims

1. Use of a substance with oxytocin activity for the preparation of a pharmaceutical composition in order to improve cell regeneration, such as to treat infertility *in vitro* and *in vivo* and peripheral and central neuropathies.

2. Use according to Claim 1, **characterized** in that the substance is selected from the group consisting of the following compounds:

Cys- V -W-X-Asn-Cys-Pro-Y-Z-NH_2

wherein

V is selected from the group consisting of Tyr and Phe

W is selected from the group consisting of Ile, Val, Hoph, Phe, and Cha,

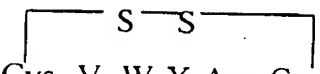
X is selected from the group consisting of Gln, Ser, Thr, Cit, Arg, and Daba,

Y is selected from the group consisting of Ile, Leu, nothing, Val, Hos, Daba, and Cit,

Z is selected from the group consisting of Gly, nothing, and Ala.

3. Use according to Claim 1-2, **characterized** in that the substance is selected from the group consisting of the following compounds: oxytocin, mesotocin, isotocin, annetocin, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

4. Pharmaceutical composition in order to improve cell regeneration, **characterized** in that it comprises an effective concentration of at least one substance with oxytocin activity in mixture or otherwise together with at least one pharmaceutically acceptable carrier or excipient, wherein the substance is selected from the group consisting of the following compounds:



wherein

V is selected from the group consisting of Tyr and Phe,
W is selected from the group consisting of Ile, Val, Hoph, Phe, and Cha,
X is selected from the group consisting of Gln, Ser, Thr, Cit, Arg, and Daba,
Y is selected from the group consisting of Ile, Leu, nothing, Val, Hos, Daba, and Cit,
Z is selected from the group consisting of Gly, nothing, and Ala;
with the exception of oxytocin.

5. Pharmaceutical composition according to Claim 4, **characterized** in that the substance is selected from the group consisting of the following compounds: mesotocin, isotocin, annetocin, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

6. Pharmaceutical composition according to Claims 4-5, **characterized** in that it comprises substances that increase the release of oxytocin and/or the number or affinity of receptors, such as estrogen, or drugs having an α_2-agonistic effect, such as clonidine.

7. Pharmaceutical composition according to Claims 4-6, **characterized** in that the effective concentration is 4-70% by weight, preferably 0.1-50% by weight.

8. Pharmaceutical composition according to Claims 4-7, **characterized** in that it is used in the healing process after a heart attack or stroke, atrophies, degenerative diseases such as Parkinson´s disease and Alzheimer´s disease, psoriasis, osteoporosis, after transplantations, balance disturbances, a rupture of the uterus, the liver, a kidney, in order to recover proliferation of blood cells (e.g. erythrocytes, leukocytes, and lymphocytes) e.g. after a cancer therapy, in order to increase the

weight in cattle, in order to increase the human senses (e.g. to improve damaged sight, hearing, olfactory sense, and taste as a result of aging), in order to relieve incontinence problems, in cosmetic applications in order to rejuvenate skin and counteract wrinkles as a result of aging, preoperatively to increase the healing capacity, and to assist in the growth of hair and nails.

9. Composition in order to promote fetal growth, **characterized** in that it comprises an effective concentration of at least one substance with oxytocin activity.

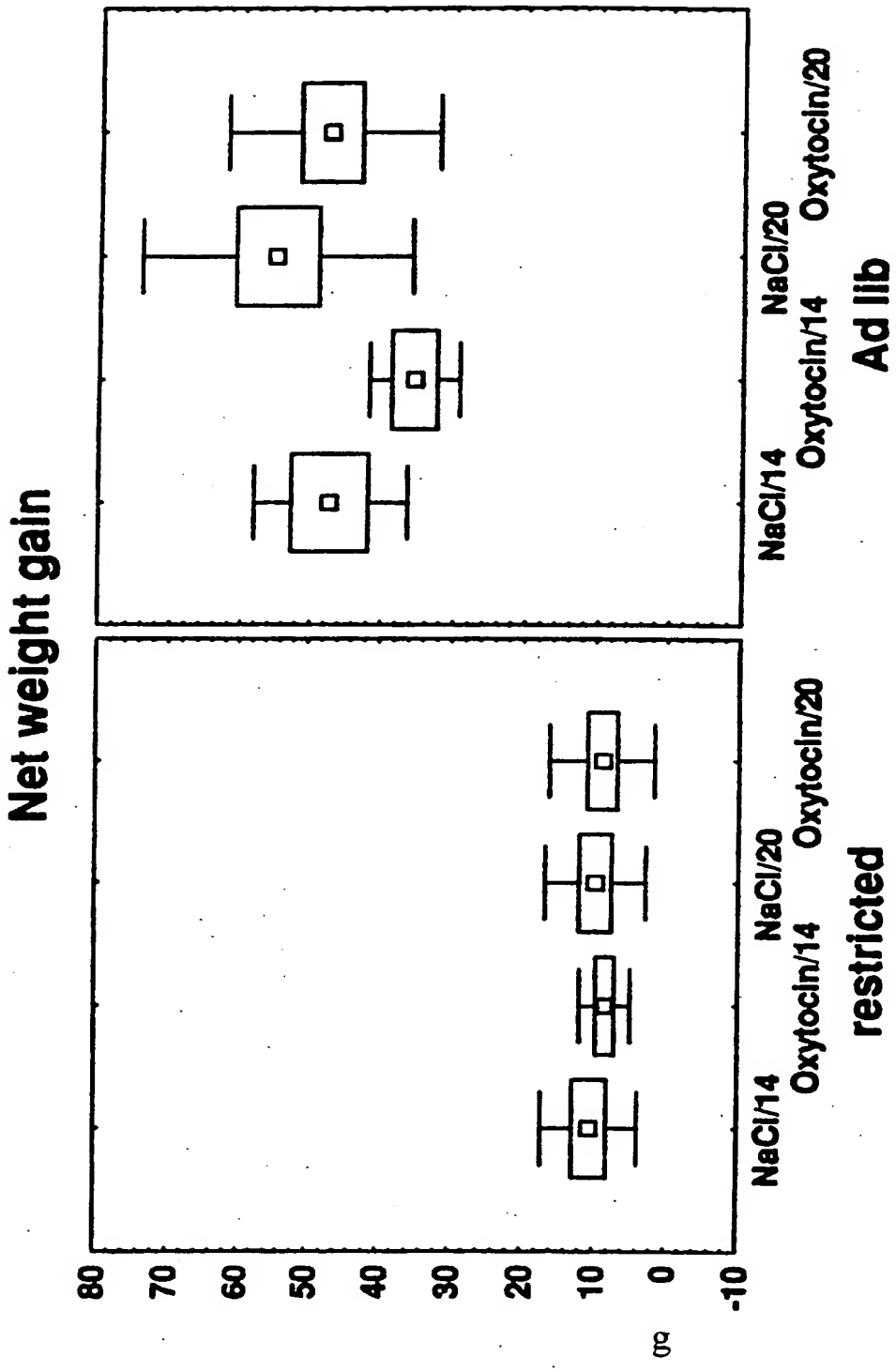


Fig. 1

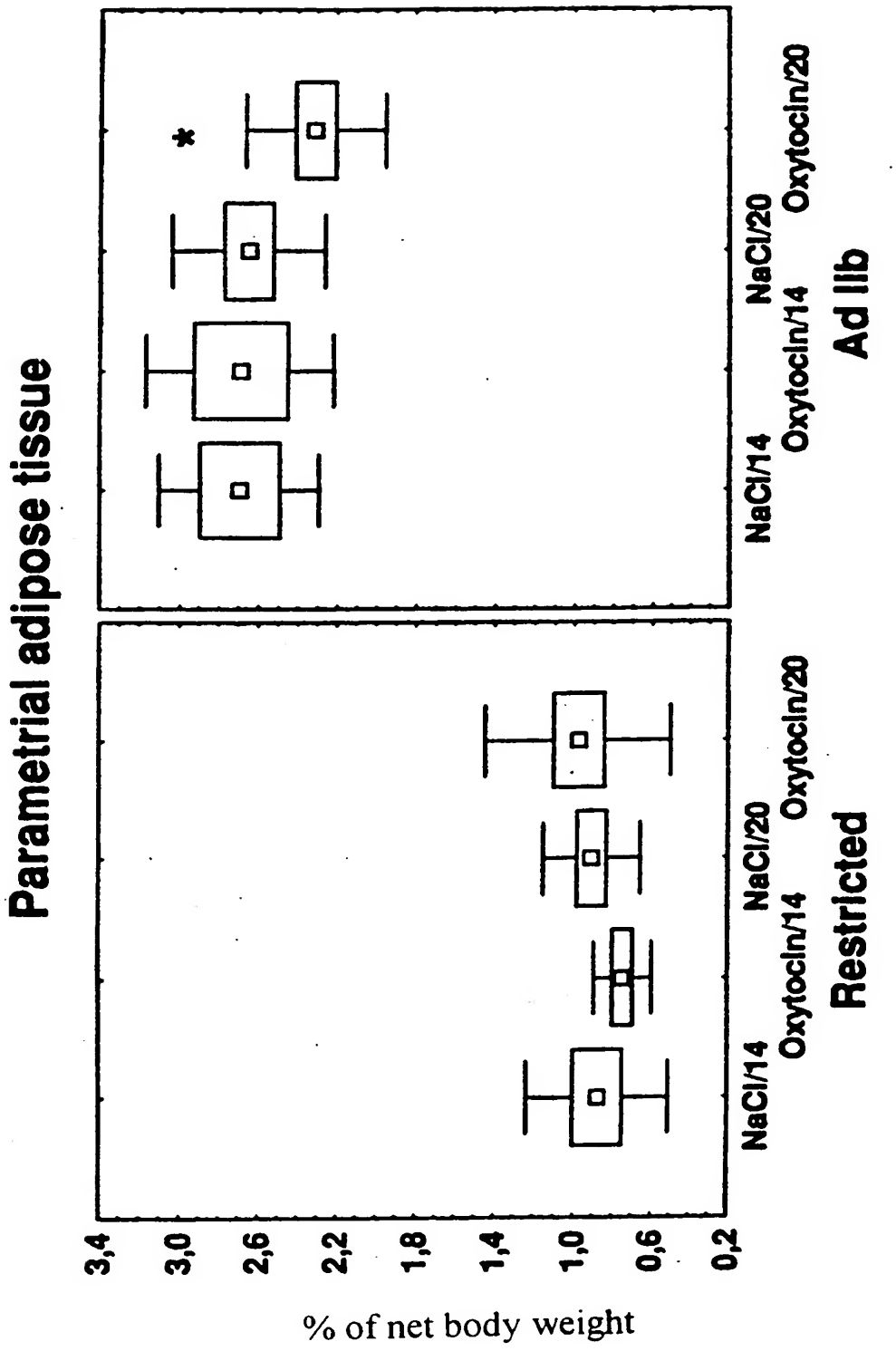


Fig. 2

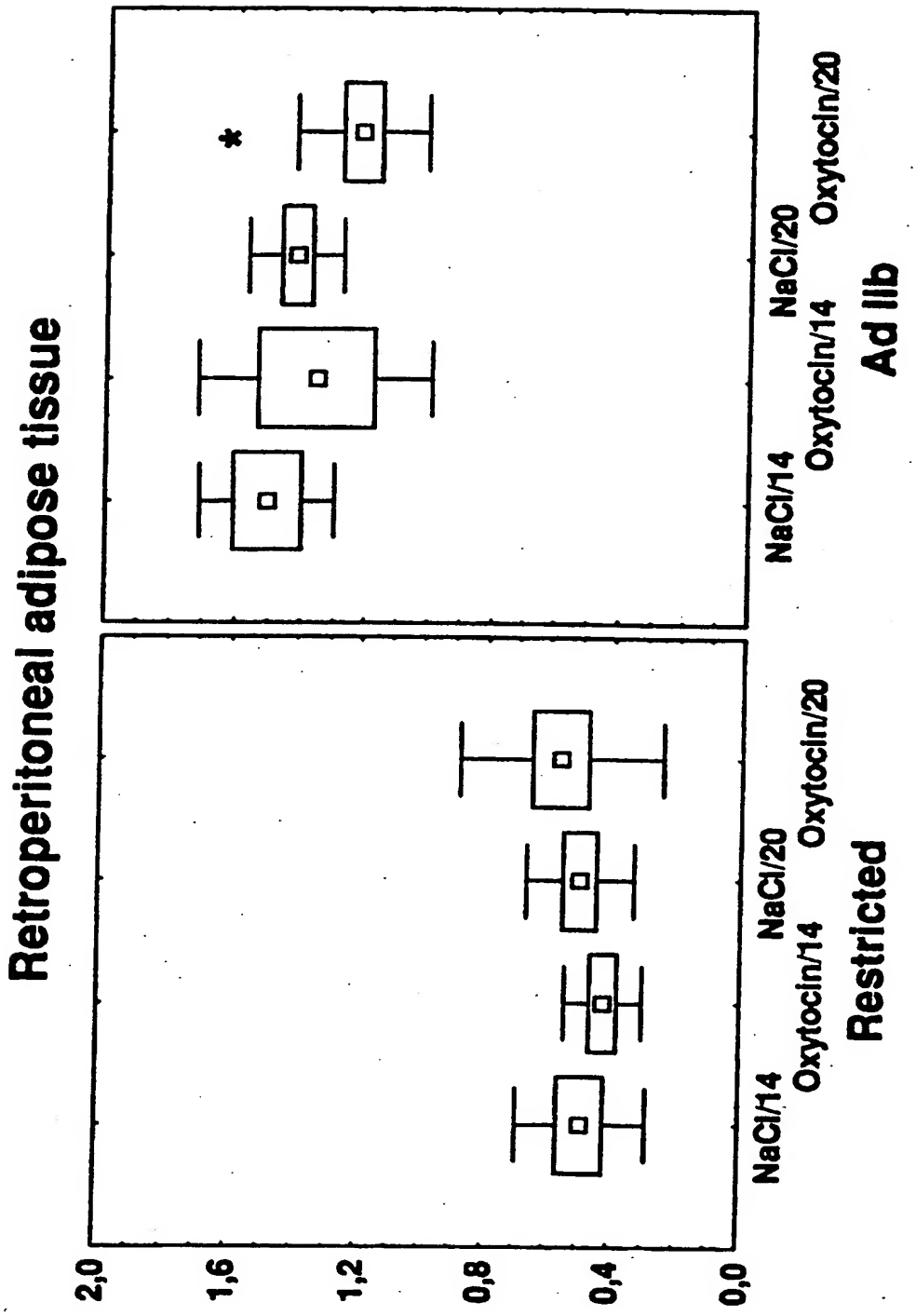


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01713

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/11, A61P 25/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, WPI, CAPLUS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Psychoneuroendocrinology, Volume 23, No 8, 1998, Kerstin Uvnäs-Moberg, "Oxytocin May Mediate the Benefits of Positive Social Interaction and Emotions" page 819 - page 835	1-9
X	Neuroscience & Biobehavioral Reviews, Volume 15, 1991, Antonio Argiolas et al, "Central Functions of Oxytocin", page 217 - page 231, see page 222	1-8
X	STN International, File CAPLUS, CAPLUS accession no. 1995:339170, Document no. 122:96599, Levay, P.F. et al: "Oxytocin: a short review", S.-Afr. Tydskr. Natuurwet. Tegnol. (1993), 12(3)	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2000	31.01.2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf/Els Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01713

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 4312913 A1 (KNAUF, SIEGFRIED), 13 October 1994 (13.10.94) -- --------	1-8

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.
PCT/SE 99/01713

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4312913 A1	13/10/94	DE 4236293 A	04/03/93
		DE 4244639 A	07/07/94
		DE 4337024 A	04/05/95

Form PCT/ISA/210 (patent family annex) (July 1992)